REMARKS

Objection to the Specification

The Examiner has required the submission of an abstract on a separate sheet under 37 CFR 1.72(b). MPEP § 608.01(b) states "the abstract for a national stage application filed under 35 U.S.C. 371 may be found on the front page of the Patent Cooperation Treaty publication (i.e., pamphlet). See MPEP § 1893.03(e)." Applicant submits that this fulfills the requirements of 37 CFR 1.72(b). If this is not sufficient, Applicant submits an additional copy of the abstract attached hereto.

Claim Rejection – 35 USC 103

Claims 1-32 are pending in the present application.

Claims 1-32 were rejected by the Examiner under 35 U.S.C. 103(a) as being unpatentable over Kayman et al. (U.S. Patent No. 5,643,756). Kayman et al. was published July 1, 1997, thus, the effective date of Kayman et al. under 35 U.S.C. 103 (a) is July 1, 1997. As stated in the attached Declaration by the Inventor under 37 CFR 1.131 ("Declaration"), the present invention was conceived of and reduced to practice prior to July 1, 1997. For this reason, Kayman et al. is not a proper reference under 35 USC 103 (a).

Furthermore, as noted in the Declaration, at the time the claimed invention was made, both the subject matter of the Kayman et al. patent, and the claimed invention, were the subject of an obligation to assign to the Public Health Research Institute of New York, Inc. (PHRI). Kayman et al. is assigned to PHRI, as is the present application (an assignment filed in U.S.S.N. 09/508,208, of which the present application is a continuation, was recorded in the U.S. Patent and Trademark Office on August 10, 2000 at Reel 011050, Frame, 0866).

For the foregoing reasons, Applicant respectfully requests withdrawal of the rejection under 35 U.S.C. 103(a), and allowance of the pending claims.

Enclosed is a check for the Petition for Extension of Time fee. Please apply any other charges or credits to deposit account 06-1050.

Respectfully submitted,

Date: 9/23/2003

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Attorney Docket No.: 07763-048002 #12

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

2003 SEP 22 PM 4:15

Applicant : Abraham Pinter
Serial No. : 10/038,407
Filed : January 2, 2002
Title : HIV-1 GP120 V1/V2 DOMAIN EPITOPES CAPABLE OF GENERATING NEUTRALIZING ANTIBODIES

Art Unit : 1648
Examiner : Park

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION BY THE INVENTOR UNDER 1.131

I, Abraham Pinter, a citizen of the United States, residing at 1250 East 22nd Street Brooklyn, New York 11210, hereby declare as follows:

1. I am the inventor of the subject matter disclosed and claimed in the above-referenced United States Patent Application.

2. I am familiar with the present claims of the application, which are directed to, *inter alia*, proteins that (1) include a gp120 V1/V2 domain of an HIV-1 strain; (2) do not include the gp120 V3 domain of an HIV-1 strain; (3) do not substantially bind CD4; and (4) the gp120 V1/V2 domain displays an epitope recognized by an antibody that neutralizes at least one HIV-1 primary isolate with an ND_{90} of less than 100 µg/ml; nucleic acid molecules encoding the proteins; and the use of the proteins as vaccines. Also included are monoclonal antibodies that bind the V1/V2 domain of an HIV-1 strain Case-A2 and neutralize at least one clade B HIV-1 primary isolate and at least one clade D HIV-1 primary isolate with an ND90 of less than 100 µg/ml.

3. I have reviewed the Office Action mailed 03/24/2003, and I am familiar with Kayman et al., U.S. Patent No. 5,643,756. I am an inventor on that patent as well. At the time the claimed invention was made, both the subject matter of the Kayman et al. patent, and the

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CERTIFICATE OF MAILING BY FIRST CLASS MAIL

I hereby certify under 37 CFR §1.8(a) that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

23 Sept. 2003
Date of Deposit

Diane C. Brown
Signature

Diane C. BROWN
Typed or Printed Name of Person Signing Certificate

claimed invention, were subject to an obligation to assign to my employer, the Public Health Research Institute of New York.

4. Prior to July 1, 1997, I had completed my invention as described and claimed in the above-identified application in this country, a NAFTA country or WTO country, as evidenced below.

5. I submit herewith Exhibit A, evidence showing conception and reduction to practice of the claimed invention prior to July 1, 1997. This Exhibit is excerpted from a funding report filed prior to July 1, 1997.

Prior to July 1, 1997, I had conceived of a peptide including the V1/V2 domain of HIV gp120, but not the V3 domain or the CD4 binding domain, and the use of such a peptide as an immunogen useful in vaccinating subjects to induce an immune response capable of neutralizing a primary isolate of HIV.

- Exhibit A, page 7 describes experiments in which human anti-V1/V2 antibodies were isolated, to evaluate their potential to neutralize primary virus isolates. Serum from several HIV-infected individuals was used. Antibodies directed against linear, type-specific V1 sequences, as well as several distinct conserved, conformational V1/V2 epitopes, were isolated by affinity purification (see Figure 1).

- Figures 2 and 3 on page 8 illustrate the neutralizing potential of the human anti-V1/V2 conformational antibodies and the anti-V1 linear sequence antibodies. Table 2 summarizes the data illustrated in Figures 2 and 3; demonstrating that the antibodies against the V1/V2 conformational epitopes, recovered by elution with pH 1 or GuHCl, had potent neutralizing activity.

- Section A-3., on page 9, describes the analysis of the clade-specificity of the human anti-V1/V2 conformational antibodies. Table 3 presents some representative data; as is shown in Table 3, NA#7, a typical North American serum, reacts strongly with sequences from isolates from North American and Brazilian clade B isolates, as well as sequences from clade E Thai isolates As discussed in section 4 on the bottom of page 9, human anti-V1/V2 conformational antibodies also exhibit cross-clade neutralization activity. This is very surprising, given the hypervariable nature of the V1/V2 domain.

- On page 11 of Exhibit A, Section B-1., the idea of an HIV vaccine based on a V1/V2 sequence, such as is claimed in the present application, is discussed. The text describes the use of fusion proteins based on the V1/V2 domain of gp120 that are encompassed by claim 1. These fusion proteins contain V1/V2, but do not contain the V3 domain, nor do they bind CD4, as they do not contain the CD4-binding epitopes. The V1/V2 sequences are derived from the Case-A2 strain of HIV-1, which contains essentially the North American clade B consensus V2 sequence. In fact, the sequences shown on page 12, Figure 6, encompass the protein sequences recited in claims 11-16 and 32. More specifically, the sequence shown in the diagram on the lower left of Figure 6 encompasses the full version of V1/V2, with three disulfide bonds, as claimed in claim 32. The sequence shown in the diagram on the lower right of Figure 6 literally illustrates SEQ ID NO: 1, e.g., as recited in claims 14-16, and this sequence encompasses the sequences recited in claims 11-13. Claim 17 recites that the protein is a glycoprotein; this is also illustrated in Figure 6. Both of the sequences shown in Figure 6 illustrate proteins that include the V1/V2 domain of HIV-1 gp120, do not include the V3 domain, and do not bind CD4, as recited in claim 1.

- In sections B-2. and 3., pages 12-14 of Exhibit 1, experiments involving immunizing rats with purified V1/V2 fusion proteins are discussed. As recited in claim 21, the rats were stimulated to form neutralizing antibodies by immunization with fusion proteins that include the V1/V2 domain of HIV-1 gp120, do not include the V3 domain, and do not bind CD4 (e.g., proteins as recited in claim 1). As recited in claims 22-23, the V1/V2 fusion proteins were suspended in a carrier, and formulated with an adjuvant. The fusion proteins were administered to the rats by injection (as recited in claim 26). The rats successfully raised cross-reactive antibodies to the fusion protein, the majority of which were directed against a particular epitope, the sequence of which we determined, as illustrated in Figure 7 on page 13. This epitope sequence is encompassed by the sequences recited in each of claims 11-16.

- The neutralization activity of antibodies raised by rats immunized with the V1/V2 fusion protein was evaluated as well, and the results are described in section B-4., pages 14-15. As illustrated in Fig. 9 and discussed on page 15, these antibodies neutralize at least one HIV-1 primary isolate with a ND_{90} of less than 100 µg/ml, as recited in Claim 1. For example, the top of page 15 reads:

  "The V1/V2-specific antibodies possessed potent neutralization activities for all four viruses tested, the T-tropic NL-HX and M-tropic NL-HX-ADA, Ba-L and ADA isolates (Fig. 9). ... [T]he residual V1/V2-specific antibodies neutralized [50% of the viral infectivity] in the range of 0.01-0.1 µg/ml."

Both Ba-L and ADA are primary HIV-1 isolates, and the ND_{50} data, with ranges of less than 0.1 µg/ml, suggests that both are neutralized with a ND_{90} of less than 100 µg/ml. Therefore, to summarize, Exhibit A describes fusion proteins that include the V1/V2 domain of HIV-1 gp120, do not include the V3 domain, and do not bind CD4, and display epitopes that are recognized by antibodies that neutralize at least one HIV-1 primary isolate with a ND_{90} of less than 100 µg/ml.

6. Thus, Exhibit A demonstrates that I had conceived of the use of a V1/V2 domain-derived peptide as an HIV vaccine immunogen prior to July 1, 1997. In sum, I submit evidence herewith that shows conception and reduction to practice of the claimed invention prior to July 1, 1997.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, under Title 18 §1001 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Sept. 22, 2003
Date

Abraham Pinter
Abraham Pinter

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[redacted]

BODY

A. CHARACTERIZATION OF EPITOPES MEDIATING EFFICIENT NEUTRALIZATION OF PRIMARY HIV-1 ISOLATES

1. Potent neutralizing activity of an anti V2 MAb for both T-tropic and M-tropic isolates of HIV-1 (3, 8, 9)

Numerous studies have indicated that primary, macrophage-tropic (M-tropic) isolates of human immunodeficiency virus type 1 (HIV-1) are considerably more resistant to neutralization by monoclonal antibodies (MAbs) and polyclonal human antisera than lab-adapted T cell-tropic (T-tropic) strains. This effect was examined in a closely matched pair of molecularly cloned prototypical T-tropic (NL-HX) and M-tropic (NL-HX-ADA) isolates. NL-HX contained the HXB2 *env* while in NL-HX-ADA the V3-V5 domain of NL-HX was replaced by the equivalent region of ADA virus, a typical macrophage-tropic primary ioslate. Thus, these two viruses contained large regions of sequence identity in the *env* gene, including identical V1/V2 domains and gp41 sequences.

Using a panel of MAbs directed against HIV-1 envelope proteins previously reported to possess potent neutralizing activities we found that whereas most of the MAbs exhibited a large preference for neutralization of the T-tropic isolate (ND_{50} ratios ranging from 8-800), C108G, a potently neutralizing chimpanzee MAb directed against a type-specific epitope in the V1/V2 domain of gp120, possessed very potent activity against both viruses, exhibiting only a 3-fold increase in ND_{50} and ND_{90} for the M-tropic virus (Table 1). C108G also possessed potent neutralizing activity against another primary virus, Ba-L, that contained the C108G epitope. These results indicated that the C108G epitope was a particularly sensitive neutralization target in M-tropic viruses, and suggested that the V1/V2 domain in general may be a preferential neutralization target in M-tropic viruses..

	ND_{50} (ng/ml)			ND_{90} (ng/ml)		
	NL-HX	NL-HX-ADA	NL-HX-ADA/NL-HX	NL-HX	NL-HX-ADA	NL-HX-ADA/NL-HX
C108G	2.5	7.6	3.0	20	66	3.3
2F5	5.0	4,060	812	130	-	-
IgG-b12	7.5	1,000	133	71	4,710	66.3
sCD4	14	1,550	111	127	4,250	33.5
C311E	21	>>1,000	>>50	90	-	-
2G12	38	900	24	560	4,800	8.6
5145A	190	>50,000	>263	950	-	-
447D	270	2,125	8	840	4,600	5.5

Table 1. 50% and 90% end points and end point ratios for neutralization of T-tropic (NL-HX) and M-tropic (NL-HX-ADA) clones of HIV-1 by a panel of monoclonal antibodies (- indicates samples for which end point were not achieved). MAbs used all had been previously reported to possess potent neutralizing activity for HIV-1, and were directed against epitopes in V2 (C108G), V3 (C311E and 447D), CD4-binding site (sCD4, 5145A, IgG-b12), gp41 (2F5), and undefined epitope in gp120 (2G12).

2- Isolation and characterizati n of human anti-V1/V2 antibodies with potent neutralizing activities f r primary viruses (3, 8)

We had previously shown that the sera of fraction of HIV-1-infected humans contain detectable antibodies directed against the V1/V2 domain of gp120 (4). In order to determine whether these antibodies also possessed neutralizing activities for primary viruses, V1/V2-specific antibodies were isolated from several human sera and used in neutralization assays. These experiments were performed initially with the serum of two laboratory worker infected with the IIIB strain of HIV (12). These cases provided us with patients for whom viruses with autologous V1/V2 sequences were available, allowing us to examine both type-specific and crossreactive neutralizing activities. ELISA assays using synthetic proteins and a variety of V1/V2 fusion glycoproteins demonstrated that the serum of one infected lab worker, LWS, contained antibodies directed against both linear, type-specific V1 epitopes, and conserved, conformational V1/V2 epitopes, while the sera of a second infected lab worker, LWF, contained antibodies directed primarily against a linear V1 sequence. These antibodies were fractionated by affinity chromatography on columns to which either the autologous V1 peptide (KNDTNTNSSSGRMIMEKGEIK) or the autologous HXB2 V1/V2 fusion protein had been coupled. Specific antibodies were recovered by sequentially eluting the column with pH 3, pH 1 and 5M GuHCl-containing buffers. After buffer exchange, the column eluates were characterized by ELISA against various gp120s and V1/V2 antigens and their neutralizing activities tested against several T-tropic and M-tropic viruses.

The differential elution from the V1/V2 fusion protein column allowed the fractionation of antibodies specific for distinct V1/V2 epitopes. The antibody fraction eluted with pH 3 buffer was enriched for type-specific antibodies directed against the linear V1 epitope, while more stringent elution conditions (pH 1 and 5M GuHCl) resulted in the recovery of antibodies directed against conserved conformational epitopes (Fig. 1). The antibodies eluted by low pH buffers possessed

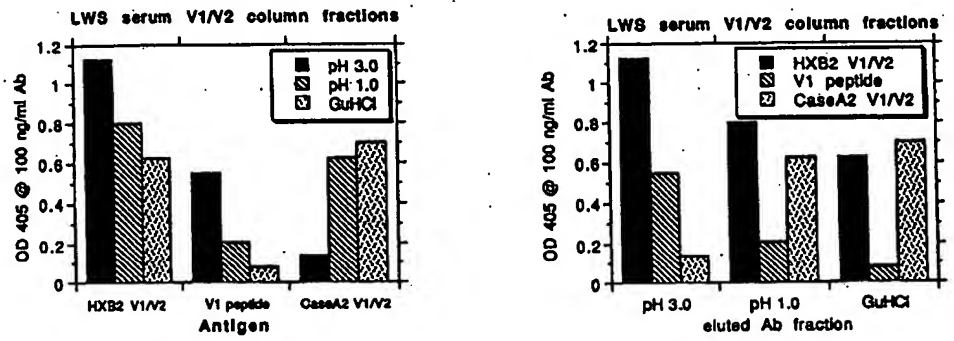

Fig. 1. Antigenic specificity of different antibody fractions generated by differential elution of V1/V2 immunoaffinity column against HXB2 V1/V2 protein, HXB2 V1 peptide and Case.A2 V1/V2 protein. ODs obtained in ELISAs for each of these antigens for an equivalent antibody concentration (100 ng/ml) are shown for each antibody fraction.

considerable neutralizing activity for both T-tropic and M-tropic viruses containing the homologous V1/V2 domains (Fig. 2). This activity was due at least in part to the anti-V1 antibodies present in these samples, since anti-V1-specific antibodies purified from both this serum and from LWF serum by immunoaffinity chromatography on a V1 peptide derived from the HXB2 sequence also neutralized both HXB2 and NL-HX-ADA, an M-tropic virus with the homologous V1/V2 domain, but not viruses with a heterologous V1/V2 domain (Fig. 3).

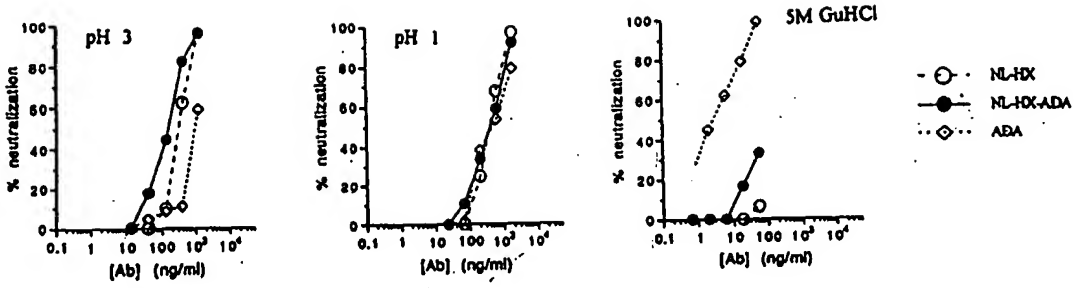

Fig. 2. Neutralization curves for NL-HX, NL-HX-ADA and ADA by immunoaffinity-purified anti-V1/V2 antibodies from LWS serum. pH 3, pH 1 and 5M GuHCl eluates of HXB2 V1/V2 column were tested for neutralization against NL-HX, NL-HX-ADA and ADA viruses, as indicated in the legend.

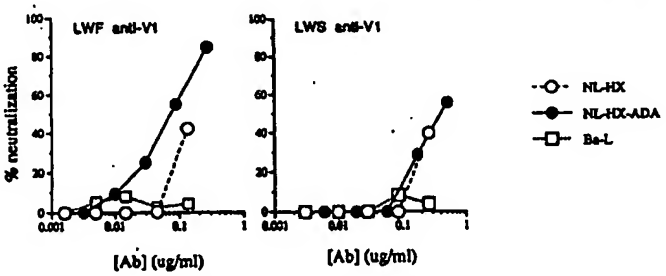

Fig. 3. Neutralization curves for NL-HX, NL-HX-ADA and Ba-L by anti-V1 antibody fractions of LWS and LWF serum purified by immunoaffinity chromatography on a V1 peptide column.

The antibodies to conserved epitopes recovered in the GuHCl eluates possessed potent neutralizing activities against both related and unrelated M-tropic viruses (Fig. 2 and Table 2). These antibodies possessed more potent neutralizing activity for these primary viruses than any previously described monoclonal or polyclonal antibodies. These results indicated that the V1/V2 domain of HIV-1 gp120 contains both type-specific and conserved epitopes that are particularly sensitive neutralization targets in macrophage-tropic viruses, and that this region is the major neutralization domain in primary viruses for at least one human serum. These findings suggested that vaccines that induce antibodies against the conserved neutralization epitopes in this region may provide effective protection against HIV-1.

Antibody fraction	Virus	ND25 (ng/ml)	ND50 (ng/ml)	ND90 (ng/ml)
pH 3	NL-HX	200	300	990
	NL-HX-ADA	65	155	775
	ADA	600	900	>1,100
pH 1	NL-HX	200	400	1,475
	NL-HX-ADA	150	400	1,700
	ADA	150	400	>1,800
5M GuHCl	NL-HX	>>53.5	>>53.5	-
	NL-HX-ADA	35	>53.5	-
	ADA	0.8	3	36

Table 2. Neutralization end points of various anti-V1/V2 antibody fractions eluted from V1/V2 affinity column.

3. Human sera contain antibodies directed against V1/V2 epitopes that are conserved across clades

Previous studies with monoclonal antibodies and human antisera demonstrated the presence of epitopes in V1/V2 that were conserved for many clade B gp120 isolates. In order to obtain more information about the breadth of the conservedness of these epitopes, we expressed the V1/V2 domains of a number of additional clade B and non-clade B sequences, including several Brazilian and Thailand clade B sequences, and two Thai clade E sequences, 92TH006 and 92TH022 (1) (Table III). A typical North American human serum, #7, from a clade B-infected individual, reacted strongly with all of the sequences, including those from the clade E Thai isolates. In contrast, mouse mab SC258 (7) recognized all the clade B but not the clade E sequences, while human mab, 697D (2), previously reported to recognize a broadly conserved epitope, reacted only with the North American clade B sequences. Similar broad crossreactivity was observed for a number of additional human antisera. This analysis provides further evidence for the presence of multiple epitopes in the V1/V2 domain, with vary in their degrees of conservedness, and demonstrates that a number of human sera contain antibodies which recognize V1/V2 epitopes conserved across clades.

		Antibody tested		
Viral origin	Viral clone	SC258	697D	NA#7
NA clade B	CaseA2	+++	+++	+++
Brazil clade B	Br.B.14.06	+	-	+++
Brazil clade B	Br.B.20.04	++	-	+++
Thailand clade B	Th.B.14.12	+++	-	++
Thailand clade B	Th.B.26.06	+	-	+++
Thailand clade E	Th.E.06.5	-	-	+++
Thailand clade E	Th.E.22.4	-	-	+++

Table III. Variation in reactivity of MAbs and a human serum for different V1/V2 sequences

4. Analysis of cross-clade neutralization activity of human antibodies to V1/V2 epitopes

In collaboration with John Mascola at the Walter Reed Army Institute for AIDS Research, we have examined the anti-V1/V2 reactivity of a panel of sera from HIV-infected patients in Thailand. The major HIV type currently found in Thailand is clade E, while clade B viruses are found less frequ ntly (5). A panel of 8 clade E and 2 clade B Thai sera was used in the initial analysis. Several of the clade E sera recognized both clade E V1/V2 sequences and two North American clade B V1/V2 proteins tested, derived from the HXB2 and CaseA2 sequences. This demonstrates that despite the considerable difference in their primary sequences, V1/V2 domains from different clades share epitopes.

The presence of conserved V1/V2 epitopes recognized by human sera raised the possibility that these may be mediating cross-clade neutralization. This was examined by purifying the V1/V2-specific antibodies from a pool of three Thai plasma samples which contained antibodies reactive with clade B V1/V2 fusion proteins, and examining their neutralizing activities for clade B viruses. The sera were pooled before we learned the clade classification of the sera, and consisted of two clade E and one clade B sera. The sera were fractionated on a column containing immobilized Case-A2 V1/V2 fusion protein (10). The Case-A2 isolate was selected because it contained the clade B consensus sequence in the major region of V2 (see below).

The pooled sera possessed considerable neutralizing activity against one T-tropic virus (NL-HX) and all three M-tropic viruses (NL-HX-ADA, Ba-L and ADA) tested. After fractionation on a Case-A2 V1/V2 column a total of ~50 μg of specific antibody was recovered from an initial sample of 11.2 mg of total IgG. Most of these antibodies were in the pH 1 fraction (28 μg), with 13.5 μg in the pH 3 fraction, 4.9 μg in the 5M GuHCl fraction and 4.7 μg in the 8M GuHCl fraction. All of these fractions possessed strong neutralization activities against all three M-tropic viruses tested, with the two GuHCl fractions possessing the most potent activities (Fig. 4). The ND_{50}s of the 8M GuHCl fraction were lower for both ADA (45 ng/ml) and Ba-L (< 63 ng/ml) than for NL-HX-ADA (180 ng/ml).

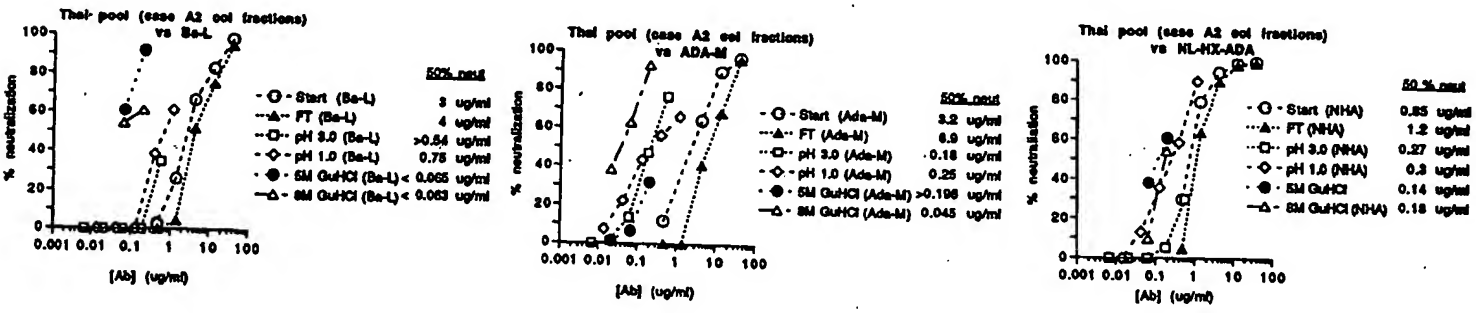

Fig. 4. Neutralization of clade B macrophage-tropic HIV-1 isolates by anti-V1/V2 antibodies purified from a pool of three Thai sera.

Interestingly, when these antibody fractions were tested against the T-tropic virus, NL-HX, they all enhanced infectivity (Fig. 5). The numbers of infected cell detected in the presence of these antibodies increased with increasing antibody concentrations; the extent of enhancement ranged from a 2.5-fold increase over control for 1.1 μg/ml of the pH 1 fraction to a 5-fold increase for 0.2 μg/ml of the 5M GuHCl fraction. These antibodies were isolated from a pool of three Thai plasma which were derived from two subjects infected with clade E viruses and one with a clade B virus, and thus it was not clear which plasma sample contained the neutralizing and enhancing activities. We are presently repeating these experiments using individual samples to determine which of these activities are due to cross-clade antibodies. The antigenic specificity, generality and mechanism of this enhancement, and the nature of epitopes mediating this effect, will be the subject of further research during the coming year.

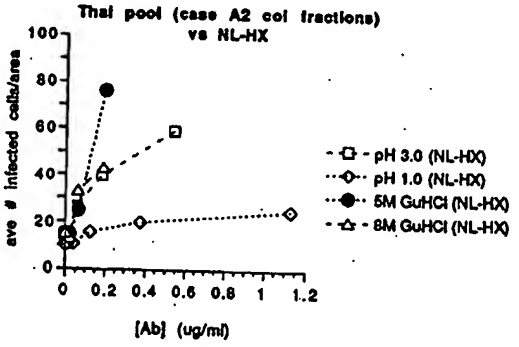

Fig. 5. Enhancement of a T-tropic clade B HIV-1 isolate by anti-V1/V2 antibodies purified from a Thai sera pool.

5. Synergistic neutralization between an anti-V2 MAb and MAbs to the V3 loop and CD4-binding site (9)

Previous studies have indicated that certain combinations of antibodies directed against the V3 loop and the CD4-binding site possessed synergistic neutralizing activities for T-tropic viruses. In order to see whether such synergy was also seen for an anti-V2 antibody and whether it extended to primary viruses, the effect of combining C108G with antibodies to the V3 loop and the CD4 binding site on HIV-1 neutralization was examined. Synergistic neutralization of HIV-1 was observed for these antibody combinations against both T-tropic and M-tropic isolates. Extent of synergy was quantitated by calculation of combinition indices (CI). Significant synergy with a given MAb pair was seen over a range of MAb ratios with the optimal effect centering around the ratio at which the MAbs were equipotent for a given HIV isolate. Synergism in all cases was greater against heterogeneous isolates (IIIB and Ba-L) than against clonal isolates (HXB2 and NL-HX-ADA), suggesting that in the former case, strain broadening was a contributing factor to the synergism. A three-MAb combination consisting of C108G, an anti-V3 MAb and an anti-CD4 binding site MAb, was more effective in neutralizing HXB2 than any of the two MAb combinations. These results suggest that vaccines that induce combinations of antibodies against all three of these major neutralizing domains may have more potent activities than those that induce antibodies against only a single domain.

B. DEVELOPMENT OF A V1/V2-BASED VACCINE AGAINST HIV-1

1. Identification and expression of an efficiently folded V1/V2 fusion protein that contains the North American clade B V2 consensus sequence.

The studies described above indicate that epitopes present in the V1/V2 domain of HIV-1 gp120 can mediate potent neutralization of primary isolates of HIV-1. As a result of these results, we were interested in developing an HIV-1 vaccine based on V1/V2 fusion proteins similar to the HXB2 protein described in the above studies. One difficulty encountered towards this approach was that the V1/V2 domain is highly conformational, containing three disulfide bonds, and a large fraction of the HXB2 V1/V2 fusion protein was misfolded (13). In addition, the HXB2 sequence contained several rare substitutions at several polymorphic sites in V2, which resulted in the presence of a number of type-specific epitopes (6, 7, 13). We therefore searched for another V1/V2 sequence that contained a more representative V2 sequence and which folded more efficiently. Wang et al. have characterized the V1/V2 domains of 57 HIV-1 sequences obtained from a group of 47 HIV-infected individuals (10). Among these sequences was the Case-A2 isolate, obtained from an infected infant, that possessed essentially the North American clade B consensus V2 sequence. We have expressed the V1/V2 domain of this isolate as a gp70 fusion protein in the Celltech pEE14 vector and have examined its utility as an immunogen.

The folding efficiency of the CaseA2 V1/V2 fusion protein was first examined by performing sequential immunoprecipitations with mab SC258, a mouse MAb directed against a conserved conformational epitope in the V1/V2 domain (Fig. 6). This analysis indicated that, as we had seen for the related HXB2 protein, a large fraction of the Case-A2 V1/V2 fusion protein was misfolded. In an attempt to improve the folding of this protein, we expressed it as a smaller form (called V1/V2-B) in which the first disulfide bond present in the conserved arm, derived from constant region 1 and 2 sequences, was absent. ELISA assays and sequential immunoprecipitations demonstrated that this molecule expressed the same range of conformational epitopes as the original construct, but was

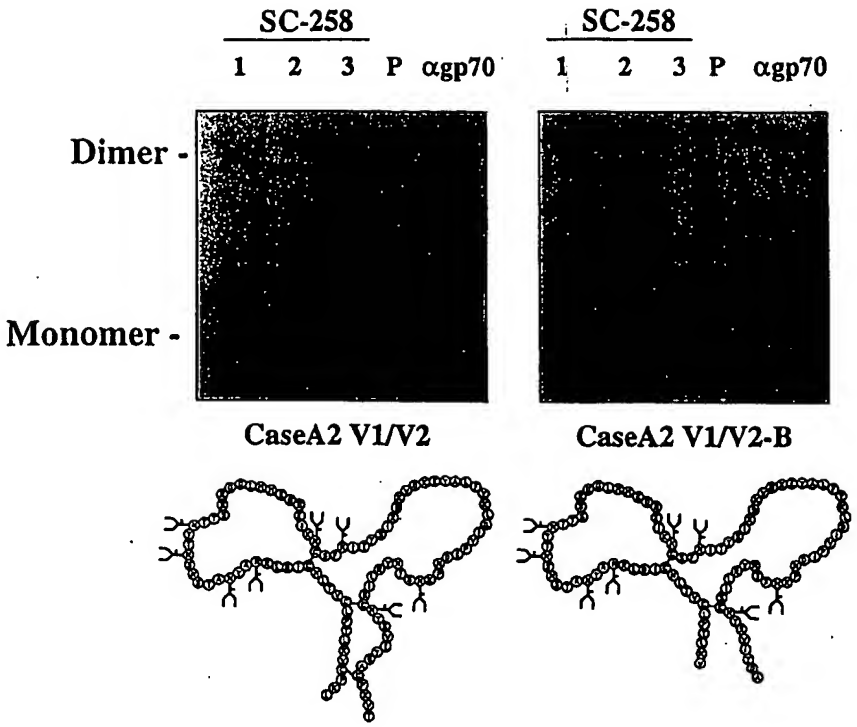

Fig. 6. Sequential immunoprecipitations of radiolabeled Case-A2 V1/V2 fusion proteins. Supernatant medium of producer cells labeled with 35S-cysteine overnight were immunoprecipitated three times sequentially with mouse monoclonal SC-258 directed against a conformational epitope followed by immunoprecipitation with goat anti-gp70 serum to detect residual fusion proteins. Samples were analyzed by SDS-PAGE under nonreducing conditions to allow visualization of disulfide-linked dimers in addition to correctly folded monomeric forms. The V1/V2 sequences present in the two form is indicated beneath the each gel analysis.

folded more efficiently. The great majority of these molecules were recognized by SC258, and no dimers were formed. Thus, the great majority of the V1/V2-B molecules were correctly folded and uniformally presented native conformational epitopes. This protein was therefore used for subsequent immunogenicity studies.

2. Immunizations of rats with purified V1/V2 fusion proteins

Initially our emphasis was to determine an efficient adjuvant and dosing schedule that elicited an efficient humoral immuneresponse to V1/V2 fusion proteins, in order to examine the specificities and functional activities of the resulting antibodies. A number of 2-4 month old female Fischer F344 rats were immunized with purified fusion glycoproteins expressing the V1/V2 sequences derived from either HXB2 or Case A2 envelope gp120. The immunogens were formulated with either QS21 or RAS MPL+TDM adjuvant in a format recommended by the manufacturers. Three rats in each group were immunized with immunogens at 15 ug/rat and were boosted in the same formulation 6 weeks later at a dosage of 1ug/rat. Rats were boosted again at a 5-6 weeks interval with the same dosage and formulation and bled 1 week post each boost, and analyzed by ELISA against various antigens.

These results showed that the Case A2 V1/V2-B protein was a more effective immunogen than the HXB2 protein. Sera from both RAS and QS21 adjuvant groups were analysed for their crossreactivity against purified envelope proteins derived from LAV, MN and CM strains, using equal

amounts of each envelope protein. In general, the group immunized with Case A2 immunogen produced higher titers and better crossreactivity than those immunized with the HXB2 protein. All three rats immunized with the Case-A2 protein produced antibodies that crossreacted with LAV gp120, and several of the immunized animal sera demonstrated appreciable titers to MN gp120, indicating that these antigens had induced antibodies crossreactive with conserved epitopes expressed on native gp120s..

3. Epitope specificity of anti-V1/V2 antibodies induced by Case-A2 V1/V2 fusion proteins

Western blots assays indicated that the sera of some rats immunized with V1/V2 fusion proteins contained antibodies that reacted with denatured V1/V2 fusion proteins. To determine whether such responses to linear epitopes were induced by immunization with the Case-A2 V1/V2 protein, and to map the epitopes recognized by such antibodies, a set of 15-mers that overlapped by approximately 5 residues and that represented the entire Case-A2 V1/V2 sequence were synthesized (Fig. 7). ELISA assays with these peptides showed that the linear epitopes recognized were localized to a single peptide, peptide 7, with the sequence SIRDKVQKEYALFYK; this corresponded to the most conserved region in the V1/V2 domain. Two other rats immunized with the Case-A2 imunogen also recognized this peptide, whereas an initial screen of a number of sera of HIV-infected humans did not identify any reactivity to this sequence. This suggested that this conserved sequence, although immunogenic when expressed in the isolated V1/V2 fusion protein, was not very immunogenic when expressed during HIV infection.

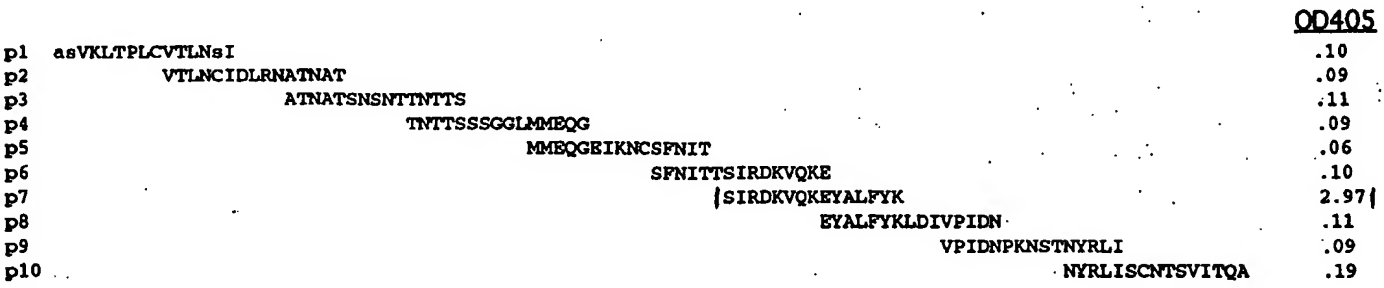

Fig. 7. ELISA reactivity of a typical serum of a rat imunized with the Case-A2 V1/V2-B fusion protein with a series of overlapping peptides derived from the Case-A2 V1/V2 sequence. Reacti vity was seen only to peptide 7.

A peptide analogue of the reactive sequence was prepared in large scale, to allow further characterization of the antibodies induced against this sequence. To increase peptide solubility and to facilitate the immobilization of the peptide two additional lysine residues were introduced at the N-terminus, followed by two naturally occurring threonines. The resulting peptide was called T15K, and has the sequence kkTTSIRDKVQKEYALFYK. The specificities of the antibodies recognizing this peptide epitopes were further defined by analyzing a series of N-terminal and C-terminal truncations of the T15K peptide (Fig. 8). Deletion of the C-terminal lysine did not affect binding of two sera, while deletion of the preceding two residues, phenylalanine and tyrosine, resulted in complete loss of bindng on one serum and partial loss of binding of the second serum. Deletion of an additional two residues, alanine and leucine, resulted in loss of binding of both sera. N-terminal deletions of the two threonines retained reactivity, while further deletion of serine and isoleucine led to the complete loss of reactivity of both sera. Thus, the minimal epitope was (I)RDKVQKEYA(LF), with the necessity of the terminal residues in parentheses

variable or unknown. Interestingly, this sequence partially overlapped with the homologous peptide determinant of C108G, STSIRGKV (11), suggesting that this region may also be a neutralization epitope.

		OD405 rat #1	OD405 rat #2
T15K	kkTTSIRDKVQKEYALFYK	**1.67**	**1.71**
pep1	kkTTSIRDKVQKEYALFY	**1.54**	**1.74**
pep2	kkTTSIRDKVQKEYAL	0.03	**0.64**
pep3	kkTTSIRDKVQKEY	0.00	0.03
pep4	kk--SIRDKVQKEYALFYK	**0.86**	**1.14**
pep5	kk----RDKVQKEYALFYK	0.00	0.17
pep6	kk-----DKVQKEYALFYK	0.00	0.07
pep7	kk------KVQKEYALFYK	0.00	0.04
pep8	kk-------VQKEYALFYK	0.00	0.00
pep9	kk---------KEYALFYK	0.02	0.00

Fig. 8. ELISA reactivity of sera of two rats immunized with the Case-A2 V1/V2-B protein to a panel of smaller peptides related to peptide 7 of the Case-A2 V1/V2 sequence.

4. Neutralization activities of anti-V1/V2 antibodies produced by immunized rats

Preliminary experiments indicated that the sera of all of the immunized rats possessed neutralization activities against a number of HIV-1 isolates. In order to evaluate the role and potency of the anti-V1/V2 antibodies present in these sera, and to eliminate background effects to other components present in the rat sera, the IgG fraction of these sera was first isolated on a protein G column and the purified immunoglobulins were then further fractionated by sequential affinity chromatography on a T15K V2 peptide column followed by a Case-A2 V1/V2-B column. In this way, we were able to separate the peptide-specific antibodies from additional antibodies that may be reactive with conformational epitopes. Specifically bound antibodies were eluted from both columns; in both cases, activity was detected only in the pH 3 eluates. The antibody concentrations of these two samples were determined by a antibody capture immunoassay specific for rat IgG; the yield of the peptide-specific antibodies was 25 µg/ml, while the V1/V2 column eluate contained only 2.9 µg/ml.. The neutralizing activities of these samples were compared to those of the starting serum and protein G-purified IgG sample.

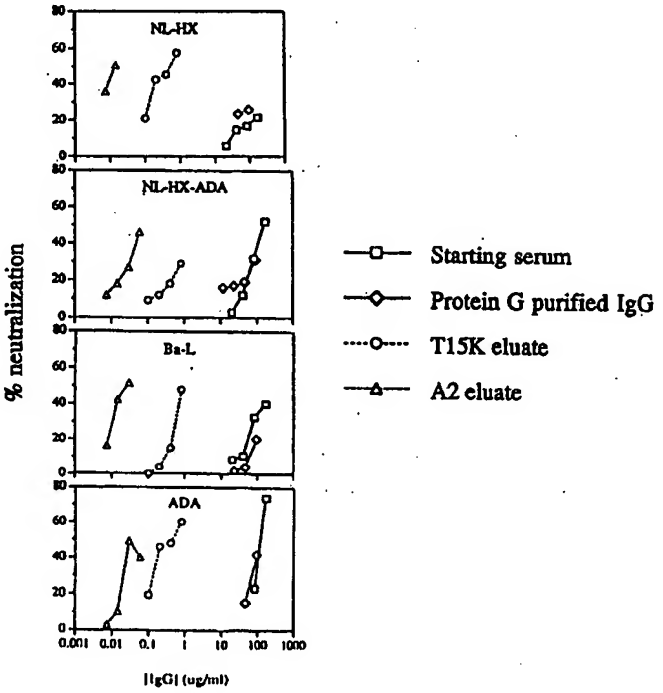

Fig. 9. Neutralizationof T-tropic and M-tropic HIV-1 isolates by sera and purified immunoglobulin fractions of a rat immunized with the Case-A2 V1/V2-B fusion protein.

The V1/V2-specific antibodies possessed potent neutralization activities for all four viruses tested, the T-tropic NL-HX and M-tropic NL-HX-ADA, Ba-L and ADA isolates (Fig. 9). The amount of antibody available for these assays was limiting, so the dilution used were not sufficient to achieve complete neutralization; nonetheless, in many cases we were able to obtain 50% neutralization end points. Whereas the starting serum and protein G IgG fraction neutralized in the general rangeof 100 μg/ml, the T15K-specific antibodies neutralized in the range of 0.1-1 μg/ml, while the residual V1/V2-specific antibodies neutralized in the range of 0.01-0.1 μg/ml. This activity was as potent as that seen for the best human anti-V1/V2 antibody fractions. Perhaps more importantly, these antibodies neutralized all of the viruses tested, including the T-tropic virus and three M-tropic viruses. This broad neutralization suggests that these antibodies may be effective against a broad range of primary viruses and clinical isolates.

CONCLUSIONS

The results obtained during the first year of this grant and summarized above indicate the importance of the V1/V2 domain in the neutralization of primary viruses and macrophage-tropic isolates of HIV-1. We have demonstrated that a V1/V2-specific monoclonal antibody possesses unusually potent neutralizing activity for both T-tropic and M-tropic isolates that bear this epitope and we have shown that a number of human sera contain anti-V1/V2 antibodies that possess broad crossreactivity and potent neutralizing activities against a number of different HIV-1 isolates, including primary isolates and viruses from distant clades. We have also found in several cases the presence of crossreactive anti-V1/V2 antibodies that have little neutralizing activity and in some cases, that enhance infectivity of HIV. We have also made progress towards the development of a candidate V1/V2-based vaccine, and have prepared an prototypical immunogen that induces broadly reactive, potent neutralizing antibodies for a number of HIV isolates, including macrophage-tropic viruses. As a result, we plan on continuing these studies during the coming year in the following directions.

1. Characterization of the protective humoral immune response in infected humans

During the coming year we will continue to characterize the humoral responses of HIV-1-infected individuals, with continued emphasis on defining the nature of antibodies capable of mediating broad neutralization of primary isolates of HIV. In addition to characterizing the V1/V2-specific antibodies present in these sera, we will use related techniques to analyze the anti-V3 antibodies and to look for the presence of antibodies that may be specific for more complex epitopes formed by sequences present in multiple domains. Emphasis will be placed on examining the cross-clade reactivity and neutralization potency of anti-V1/V2 antibodies present in the sera of infected people from various geographical areas. In particular, the presence of anti-V1/V2 antibodies that enhance infectivity or that do not neutralize but block neutralizing antibodies will be examined. The epitope specificity of neutralizing and non-neutralizing anti-V1/V2 antibodies will be determined and the mechanisms of enhancement mediating by anti-V1/V2 antibodies will be explored. If we can identify and distinguish neutralizing epitopes from non-neutralizing and enhancing epitopes, we will use this information to design second generation immunogens in which the neutralization epitopes are retained while the deleterious epitopes are deleted or modified. Such immunogens may elicit more effective immune responses than vaccines based on sequences isolated from pathogenic viruses.